#6 Ja 4/21/89

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

RECEIVED
89 APR 17 PM 5:05
GROUP 180

In re Application of:	:	
Adang/Kemp	:	Group Art Unit:
Serial No. 260,574	:	Examiner:
Filed: October 20, 1988	:	
For: INSECT RESISTANT PLANTS	:	

DECLARATION OF MICHAEL J. ADANG
UNDER 37 C.F.R. SECTION 132

I, Michael J. Adang, Ph.D., state that I am an inventor hereof. I am currently employed as Associate Professor of Entomology at the University of Georgia, Athens, Georgia, and have had considerable experience in the area of plant molecular biology and manipulation of the toxin genes of Bacillus thuringiensis as shown by my Curriculum Vitae, a copy of which is attached hereto as Exhibit A.

Exhibit B is a photograph showing insect damage to a control tobacco plant as compared with a tobacco plant transformed with pH450 containing the full-length B.t. gene under the same conditions of exposure to insects.

Exhibit C is a photograph showing insect damage to a control tomato plant as compared with a tomato plant transformed with ~~pH615~~ pH450 containing the ~~truncated~~ full-length B.t. gene under the same conditions of exposure to insects. ma 4/12/89

The following discussion provides my interpretation of the data in the application relative to expression of the full-length and partial protoxin genes, as well as data in the cited references, Barton et al. (1987) Plant Physiol. 85:1103-1109, and Vaeck et al. (1987) Nature 328:33-37, and in Fischoff et al. (1988) Biotechnology 5:807-813 and data developed in my laboratory not included in the specification hereof, all with reference to the question of whether or not the cited references show that success is reasonably predictable in achieving expression of particular B.t. genes in particular plants such that the plants are insecticidal but the B.t. protein is not lethal to the plants themselves.

Table 1, in items 1-4 lists the types of full-length B.t. genes and promoters disclosed in this application as well as three published references, together with expression and toxicity levels. Item 5 of Table 1 describes later work in my laboratory using a partial B.t. gene with the same promoter used by Barton et al.

TABLE 1

1. This Application

Construct:	pH450
enhancer	none
promoter	ORF 24 (MAS)
Non-coding leader	ORF 24
B.t. gene	B.t. 73 (6.6 type) full-length
PolyA region	ORF 24
Tobacco:	Xanthi
B.t. protein:	Range from none detectable to 2 ng/mg plant protein in independently transformed and regenerated individual plants

1. (Continued)

THW toxicity:	From 0-73% mortality in 2 days

---

2. Barton et al. (1987) Plant Physiol. 85:1103-1109.

Construct:	
enhancer	CaMV 35S enhancer
promoter	CaMV 35S
Non-coding leader	AMV 5'
B.t. gene	4.5 Type gene 645 aa's and 2 proline residues, 2 stopcodons
PolyA region	nopaline synthase
Tobacco:	c.v. Havana 425
B.t. protein:	12 ng/mg plant protein (50X variation between plants)
THW toxicity:	100% kill in 2 days

Comments: They claim that the full-length gene product was toxic to the cells. Evidence was callus formation under kanamycin selection followed by tissue death during regeneration.

---

3. Vaeck et al. (1987) Nature 328:33-37.

Construct:	
enhancer	none
promoter	PTR2' (MAS)
None-coding leader	PTR2'
B.t. gene	5.3 Type 610 aa's
PolyA region	3'TL gene 7
Tobacco:	Petit Havana SR1
B.t. protein:	maximum of 40 ng/mg
THW toxicity:	75-100% in 6 days

Comments: No or little expression from a full-length construct. No reported plant toxicity. They also used bt:kan fusion constructs with success.

---

4. Fischoff et al. (1987) Biotechnology 5:807-813.

Construct:	
enhancer	none
promoter	CaMV 35S
Non-coding leader	CaMV 35S
B.t. gene	full-length 5.3 type 646 aa's 5.3 type 725 aa's
PolyA region	NOS 3'
Tomato:	VF36
B.t. protein:	No ELISA or Western: estimate 50 ng/mg from comparative B.t. protein bioassays.
THW toxicity:	For truncated B.t. genes; 100% in 4 days, 50-80% for full-length.

---

5. Adang et al. (1988) Unpublished.

Construct (pH 615):	
enhancer	CaMV 35S enhancer
promoter	CaMV 35S
Non-coding leader	AMV 5'

5. (Continued)

B.t. gene	partial 5.3 Type gene 615 aa's
PolyA region	Orf 25
Tomato:	UC82
B.t. protein:	5-45 ng/mg plant protein (10X variation between different tissues)
THW toxicity:	100% kill in 2 days
Construct (pH 615) Tobacco:	Bioassays showed that there were fewer active tobacco than tomato plants for 615 and the relative activity was lower. Protein levels were not determined. In general for all constructs we got better kill on tomato than tobacco.

NOTE TO TABLE 1: B.t.2=full-length HD1 5.3 type gene=Monsanto's B.t.k gene=Agrigenetics' B.t.k HD1 Dipel gene. The B.t.2 differs from the others by 1-2 amino acids in the non-toxic region.

To date no laboratory has expressed levels from a full-length B.t. gene equal to a truncated gene. That does not mean the full-length gene has no value. For example, one strategy for avoiding insect resistance to B.t. is to express low levels in plants so that not all insects are killed and selective pressure is reduced.

Vaeck et al. do disclose the production of B.t. protein in plants containing the full-length B.t. gene. Note in Table 2 of Vaeck et al. that using the construct designated B.t.2 containing the full-length B.t. gene, they developed a series of transgenic tobacco plants that produced approximately 1 ng B.t. protein/mg plant protein. In some samples from these plants they measured B.t. protein up to 5.5ng/mg. No toxicity to the plants was reported. Insect mortality reported was 0-20% in 6 days which, while lower than Applicant's, is significant. Surviving larvae were reduced in weight for those plants with activity. Note also that the Vaeck et al. PTR 2' is the same promoter as ORF 24 (MAS) from T-left of T-DNA. They also comment that PTR 2' produces expression levels 10-50 times lower than CaMV 35S.

In my opinion, the statement in Vaeck et al. to the effect that none of the plants transformed with the full-length B.t.2 gene produced insect killing activity above levels obtained in NPTII-expressing control plants contradicts the data in Table 2. Table 2 indicates an insecticidal effect was obtained. (Note that data for the NPTII control plants is not provided in Table 2.) In my laboratory, NPTII-expressing controls did not exhibit any insect-killing effect. Note also that Table 2 indicates 0 insecticidal effect in some cases which means there is no background level for kill data - numbers above 0 indicate true kills. I cannot explain why the B.t.2 data in Table 2 of Vaeck et al. was characterized in the text as no better than the controls, however, it is clear from the data that Vaeck did achieve insect-killing levels of full-length B.t. gene expression without plant toxicity. Vaeck et al. also reported significant weight reduction in larvae surviving after 6 days, which could translate into higher mortality after longer feeding times.

In our laboratory, bioassay and ELISA results indicated that a full-length B.t. gene could be expressed at a level sufficient for killing insects. In 1987, we did additional experiments that are relevant to the insect activity of full-length B.t. genes in

tobacco. Using a pH450 transgenic line (AF108) transformed with the full-length gene, we followed R1 progeny and the segregation of B.t. gene copies and insect toxicity. All insect toxic plants were octopine positive (octopine synthase is adjacent to B.t. in pH450 and its presence indicates the parent plants have been transformed). There was a consistent correlation between insecticidal activity and presence of the B.t. gene. The correlation was confirmed by Southern blot analysis and B.t. DNA probes. It was clear that insecticidal activity correlated with the presence of the B.t. gene.

Full-length B.t. genes were also expressed in tomatoes following the teaching of the invention under funding by the assignee hereof. The pH450 transformations and analysis for tomato were done in Dr. Andrew Binns' laboratory. B.t. levels were low but detectable by ELISA and occasionally by Western blots. Our laboratory confirmed the insecticidal activity of these plants in bioassays in 1988.

To my knowledge Barton et al. are the only authors to have reported that the full length B.t. protein is toxic to tobacco tissue. I do not know what may have caused the discrepancy between their data and others. Possibly it could be explained by the levels expressed in the plant tissues. That is, with a weak promoter such as ORF 24 or 19S and other factors that affect expression not optimized, there is no apparent adverse effect on the plant transformation/regeneration process, whereas with a strong promoter such as the CaMV 35S promoter, higher, evidently toxic, levels of expression are achieved. Nevertheless, our results as well as those of Vaeck et al. show that it is possible to achieve insecticidal levels of B.t. protein in plants using the full-length gene without plant toxicity. Optimizing the level of B.t. gene expression is a matter of ordinary skill in the art.

We have no evidence that use of strong promoters results in plant toxicity. Using the strong CaMV 35S promoter used by Barton et al. to express the truncated B.t. gene in tomato, Fischoff et al. found no plant toxicity in tomato and did achieve insecticidal levels of expression; using the same promoter, we found no plant toxicity for truncated genes in tobacco or tomato. In general, we achieved better insect kill results for tomato than for tobacco.

It is my opinion, based on the foregoing factors, that the plant toxicity observed by Barton et al. with the full-length gene and the lack of insect toxicity observed by Vaeck et al. with the full-length gene can be explained by failure to optimize the transformation method, primarily by taking into account the plant to be transformed and the strength of the promoter to be used. Optimization of these and other parameters is a matter of ordinary skill in the art once the skilled worker has been provided with the teachings of the present application.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

Date: April 12, 1989

Michael J. Adang

MICHAEL J. ADANG